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High Glucose Down-regulates Intercellular Communication in Retinal Endothelial Cells by Enhancing Degradation of Connexin 43 by a Proteasome-dependent Mechanism*

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Intercellular communication through gap junctions (GJIC) is most likely relevant to maintaining the integrity of the blood-retinal barrier. In this study, we investigated the mechanism whereby high glucose enhances degradation of connexin 43 (Cx43), thus contributing to a decrease in GJIC. The levels of Cx43 in bovine retinal endothelial cells exposed to high glucose (25 mM) decreased about 50% as compared with controls (5.5 mM glucose). Consistently, the half-life of the protein decreased from 2.3 to 1.9 h. The proteasome inhibitors MG132 and lactacystin prevented the loss of Cx43 induced by high glucose and extended Cx43 half-life. The amount of phosphorylated Cx43 increased in high glucose and after proteasome inhibition. Scrape-loading dye transfer experiments show that high glucose is associated to a decrease of 40% in GJIC. Significantly, this reduction can be reversed by proteasome inhibitors. The decrease in GJIC in cells exposed to high glucose is associated with a loss of Cx43 from the plasma membrane, as demonstrated by immunofluorescence and biotinylation of cell-surface proteins. Results indicate that increased phosphorylation of Cx43 under high glucose is the mechanism targeting Cx43 for degradation by a proteasome-dependent mechanism. Increased degradation of Cx43 and reduction of GJIC in high glucose may be of physiological importance by contributing to endothelial cell dysfunction associated with the breakdown of the blood-retinal barrier in diabetic retinopathy.

Hyperglycemia and the consequent exposure of the intracellular milieu of the retinal capillary endothelial cells to elevated blood glucose concentrations have been implicated in the pathogenesis of vascular complications in diabetes, including the breakdown of the blood-retinal barrier (1, 2). The exact biochemical and molecular mechanisms that transduce chronic hyperglycemia into micro- and macrovascular complications of the retina are not clear. However, it has been suggested that several mechanisms play a role in the pathogenesis of diabetic retinopathy; those include increased nonenzymatic glycation (3), activation of aldose reductase (4), oxidative stress (3), increased production of diacylglycerol, and stimulation of retinal protein kinase C (PKC)[1] (5). Despite the nature of such mechanisms, early cell dysfunction related to diabetic vascular complications is often associated with abnormalities in cell-cell communication and maintenance of cell homeostasis.

Gap junctions are intercellular-channels that permit the passage of small molecules such as small metabolites, ions, and second messengers (6). These channels consist of two hemichannels, called connexons, that are located in the plasma membrane of two adjacent cells. Each connexon is composed of six subunits of the protein connexin. Gap junctions have an important role in a variety of cellular processes including homeostasis, morphogenesis, cell differentiation, and growth control (7).

Three connexin subtypes are expressed in the endothelium of the blood vessels: Cx37, Cx40, and Cx43 (8). Endothelial cells rely on a network of gap junctions for intercellular communication (9, 10). Previous studies have shown that intercellular communication in endothelial cells is reduced under conditions of hyperglycemia (11). Such a decrease in intercellular communication is most likely due to a reduction of the abundance of connexin 43 (Cx43) in endothelial cells.

One of the most remarkable aspects of gap junction biosynthesis is the exceptional metabolic lability of connexins. Although the half-life of the great majority of plasma membrane proteins exceeds 24 h, connexins present a rapid turnover rate, with half-lives of only 1.5–5 h (12–14). Until the present time, two proteolytic pathways have been implicated in connexin turnover, the lysosome (14, 15) and the proteasome (13–17). The mechanisms and upstream events that lead to the choice of one degradation pathway over the other are largely unknown. It has, however, been shown that both pathways coexist and are active in a number of cell types and in a variety of experimental and pathological conditions (18–20). For example, we have previously shown that in response to specific stimuli, proteasome is involved in the degradation of phosphorylated Cx43 in lens epithelial cells (LEC) (17).

It has been widely shown that phosphorylation of Cx43 results in decreased intercellular communication through gap junctions (GJIC), probably because of a reduction in abundance at the plasma membrane (21–24). For example, it was shown that in smooth muscle cells, high glucose induced the inhibition of GJIC activity through hyperphosphorylation of Cx43 by PKC (25). On the other hand, a recent study has shown that high glucose inhibited GJIC activity by down-regulating Cx43 synthesis in rat microvascular endothelial cells (11). We have shown before that Cx43 is remarkably labile and that the

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[1] The abbreviations used are: PKC, protein kinase C; Cx43, connexin 43; LEC, lens epithelial cells; GJIC, intercellular communication through gap junctions; BREC, bovine retinal endothelial cells; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; RT-PCR, reverse transcription-PCR; P/NP, phosphorylated/nonphosphorylated.

ubiquitin proteasome pathway is involved in the degradation of Cx43 in LEC (17). Further, we have suggested that the combined action of phosphorylation and protein degradation by the proteasome pathway acts as a mechanism of decreasing intercellular communication in the LEC (17). By analogy, it is thus conceivable that a similar mechanism may occur in retinal endothelial cells.

To investigate the mechanism whereby hyperglycemia leads to decreased intercellular communication in endothelial cells, we have hypothesized that hyperglycemia may create the conditions that favor increased degradation of Cx43, presumably involving increased phosphorylation of the protein. A reduction in Cx43 half-life and a decreased abundance of Cx43 at the plasma membrane would thus account for the reduced intercellular communication observed under conditions of hyperglycemia.

EXPERIMENTAL PROCEDURES

Cell Cultures—Bovine retinas were the source of capillaries used to isolate cells for primary culture. Cow eyes were obtained from a local slaughterhouse. Primary bovine retinal endothelial cells (BREC) cultures were established from fresh calf eyes. Under sterile conditions, the retinas were isolated and washed in Dulbecco's modified Eagle's medium (DMEM), and pieces of adherent retinal pigment epithelial cells were removed. The retinas were transferred to an enzyme solution containing Pronase (100 μg/ml), collagenase (500 μg/ml), and DNase (70 μg/ml) and incubated with shaking at 37 °C for 20 min. After incubation, the retinal digest was passed through 210- and 50-μm nylon mesh, and the microvessels trapped on top of the 50-μm mesh were collected in DMEM by centrifugation. The fragments were resuspended in DMEM with 15% fetal calf serum, 20 μg/ml endothelial growth supplement (Roche Diagnostics, Mannheim, Germany), heparin (100 μg/ml), and antibiotic-antimycotic solution (Sigma), plated, and grown on fibronectin-coated dishes in low glucose DMEM at 37 °C with 5% CO_2. Cultures were passaged every 7 to 10 days. For all experiments, cultures from passages three to five were used. To determine the effect of high glucose on Cx43 expression and GJIC activity, BREC were grown in low (5.5 mM) or high (25 mM) D-glucose medium for 8 days to confluence.

Antibodies and Reagents—The rabbit polyclonal and mouse monoclonal anti-Cx43 antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA). The mouse anti-Cx43 monoclonal antibody was raised against a peptide sequence that represents amino acid residues 360–376 of Cx43. Unless otherwise noted, all other reagents were from Sigma, except MG132, which was obtained from Calbiochem (Darmstadt, Germany). 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and MG132 were dissolved in Me_2SO.

Gel Electrophoresis and Western Blotting—Cells exposed to normal and high-glucose medium were washed, collected in phosphate-buffered saline (PBS) solution by centrifugation, and cell pellets were frozen at −80 °C until use. The cells were resuspended in lysis buffer containing 10 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, and 0.5% sodium deoxycholate, supplemented with protease inhibitor mixture (Roche Diagnostics), 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 10 mM NaF, 500 μM Na_3VO_4. The lysates were sonicated six times for 3 s to completely disrupt all cells and then centrifuged at 16,000 × *g* for 15 min. The supernatants were used to determine the protein concentration by BCA reagent (Pierce) and then were denatured with Laemmli buffer.

For studies involving total cell lysates, electrophoresis was performed on 50-μg aliquots of each sample on an SDS-12% polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with rabbit polyclonal or mouse monoclonal antibodies against connexin 43. The signals were detected by ECL system (Amersham Biosciences). Films were scanned and the optical densities of the bands were measured with appropriate software. All results are representative of at least three independent experiments.

Metabolic Labeling and Immunoprecipitation—For metabolic labeling with ^{35}S, cells were rinsed with methionine-free medium prior to incubation with [^{35}S]methionine (Amersham Biosciences) 100 μCi/ml for 1 h. The cells were then chased in DMEM supplemented with 0.5 mM unlabelled methionine in the presence or absence of MG132 for 1, 2, or 4 h. The samples were resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 2.5% Triton X-100, 0.2% SDS, pH 8.3) supplemented with protease inhibitor mixture (Roche Diagnostics), protein phosphatase inhibitor set II (Calbiochem, La Jolla, CA), 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 50 mM NaF, 500 μM Na_3VO_4. Cells were lysed by sonication, and supernatants were used for immunoprecipitation using polyclonal antibodies directed against Cx43. After centrifugation at 16,000 × *g*, the supernatants were incubated with antibodies directed against Cx43 for 3 h at 4 °C, followed by incubation with protein A-Sepharose for 1 h. The samples were then centrifuged, and the protein A-Sepharose sediments were resuspended in Laemmli buffer and denatured at 37 °C for 30 min. Proteins were resolved by SDS-PAGE, and the gels were dried and autoradiographed.

Semi-quantitative Reverse Transcription (RT)-PCR Analyses—RNA extracted from BREC grown in low and high glucose medium was simultaneously determined by semi-quantitative RT-PCR using the forward primer 5′-TTAAGGATCGTGTGAAGGGAAAGAG-3′ and the reverse primer 5′-CTAGATCTCTAGGTCATCAGGCCG-3′ for amplification of Cx43, and the forward primer 5′-AAGGAGAAGCTGTGCTACGTCGCCCTGG-3′ and the reverse primer 5′-GATCTTGATCTTCATTGTGCTGGGTGCC-3′ for amplification of actin. For simultaneous amplification of Cx43 and β-actin, cDNAs were generated in the same RT reaction. To confirm that amplification was in the linear region, separate tubes containing increasing volumes of the RT reaction were used. Amplification products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Densitometry was performed at nonsaturating exposures, and the Cx43/β-actin ratios were determined.

Biotinylation of Cell-surface Proteins—The cell-surface proteins of BREC were biotinylated. Dishes of confluent BREC cells (10-cm diameter) were rinsed twice with 5 ml of ice-cold PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, followed by the addition of 3 ml of the same cold solution containing 1 mg/ml freshly added Sulfo-NHS-SS-biotin (Pierce). After 30 min at 4 °C, the medium was removed, and the plates were washed three times with PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 mM glycine. The cells were scraped in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA containing 1% Triton, 0.5% deoxycholate, 0.1% SDS and supplemented with protease inhibitor mixture, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 10 mM NaF, and 500 μM Na_3VO_4; pH 7.5). After 10–20 min on ice the cells were sonicated, and the homogenates were centrifuged at 16000 × *g* for 10 min. The protein content of the supernatants was determined, and the same quantity of protein was transferred to 1.5-ml Eppendorf microfuge tubes containing 200 μl of Neutravidin (Pierce). After 2 h of incubation at 4 °C with agitation, the beads were washed four times with RIPA buffer. The resulting pellets were resuspended in 150 μl of 2× Laemmli buffer and incubated for 1 h at 37 °C. The beads were pelleted, and the proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and blotted against Cx43 antibodies.

Immunofluorescence—BREC grown on fibronectin-coated glass coverslips were fixed with 4% paraformaldehyde in PBS. The samples were then washed with PBS, permeabilized with 1% (v/v) Triton X-100 in PBS, and blocked with goat serum (1:10) for 20 min prior to incubation with primary mouse monoclonal antibody against connexin (1:25) for 1 h at room temperature. The specimens were rinsed in PBS and mounted with Glycergel (Dako, Carpinteria, CA). All solutions were made up in 0.02% (w/v) bovine serum albumin (Sigma) containing 0.02% sodium azide (Sigma) in PBS. For controls, primary antibodies were omitted. For alkaline phosphatase treatment, the dephosphorylation reactions were carried out in lysis buffer after overnight incubation at 37 °C in the presence of alkaline phosphatase (5 units) from *Escherichia coli* (Sigma).

Cell-cell Communication Assay—BREC grown on fibronectin-coated glass coverslips were assessed for gap junction-mediated intercellular coupling as described by Musil *et al.* (14). Briefly, the culture medium from confluent cells was removed and saved. The cells were rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin, after which a 27-gauge needle was used to create multiple scrapes through the cell monolayer in the presence of Dulbecco's phosphate-buffered saline containing 0.5% rhodamine-dextran and 0.5% Lucifer yellow. After 3 min of incubation at room temperature, the culture was rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin and then incubated for an additional 8 min in the saved culture medium to allow the loaded dye to transfer to adjoining cells. The cells were then rinsed and fixed with 4% paraformaldehyde and viewed using a fluorescence microscope with a UV light source.

Statistical Analysis—Data are expressed as means ± S.D. or S.E. Comparison between groups was performed with a Student's *t* test.

RESULTS

High Glucose Leads to a Decrease in the Amount of Cx43 by a Proteasome-dependent Mechanism—It has previously been

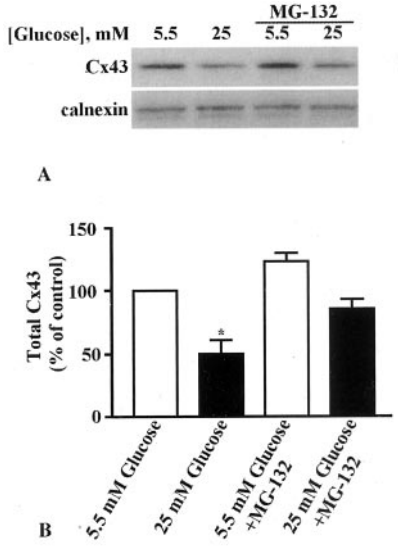

FIG. 1. **Effect of high glucose and proteasome inhibition on the amount of Cx43.** *A*, BREC were incubated in medium containing 5.5 mM (low glucose) or 25 mM glucose (high glucose) for 8 days. During the last 4 h of incubation, cells were exposed to 40 μM MG132. After the treatments, the proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with monoclonal antibodies directed against Cx43; calnexin on the same samples is included to demonstrate comparable loading of the lanes. *B*, the intensity of the bands was determined by laser scanning the films followed by quantitative densitometric analysis; results are plotted in a graph. Data are means ± S.E. (n = 3 per group). *, $p < 0.05$, difference from control incubated in low glucose as determined by unpaired two-tailed Student's t test.

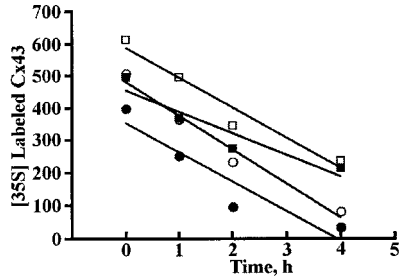

FIG. 2. **Effect of proteasome inhibitors in half-life of Cx43 under high glucose.** BREC were labeled with [^{35}S]methionine for 1 h either in the presence or absence of 40 μM MG132. The cells were then chased for 1, 2, or 4 h with non-labeled medium, and Cx43 was immunoprecipitated using a polyclonal antibody. Immunoprecipitated proteins were separated by SDS-PAGE and autoradiographed. ○, BREC grown in low glucose in the absence of MG132; □, BREC grown in low glucose in the presence of MG132; ●, BREC grown in high glucose in the absence of MG132; ■, BREC grown in high glucose in the presence of MG132. Quantification of the bands was performed using a PhosphorImager analyser (Storm 860, Amersham Biosciences). The data presented are representative of two independent experiments.

shown that hyperglycemia leads to a decrease in total Cx43 in various cell types (11, 25, 26). To investigate the mechanisms whereby high glucose leads to decreased Cx43 in BREC, the amount of total Cx43 was determined by Western blotting (Fig. 1*A*). The levels of Cx43 decreased about 50% in cells exposed to 25 mM glucose for 8 days, as compared with controls (cells incubated in 5.5 mM glucose; see Fig. 1*B*). Because the proteasome was reported to be involved in Cx43 degradation (13–17), we further evaluated the participation of the proteasome on Cx43 degradation in high glucose by treating BREC with the proteasome inhibitors MG132 (Fig. 1, *A* and *B*) or lactacystin (data not shown). Data shown in Fig. 1 clearly indicate that proteasome inhibitors prevent, to a significant extent, the loss of Cx43 induced by high glucose. In fact, the levels of Cx43 in cells exposed to high glucose were restored to about 85% of the initial levels by proteasome inhibitors (Fig. 1*B*).

Cx43 Is Stabilized by Proteasome Inhibitors under High Glucose—Because RT-PCR experiments suggest that the levels of mRNA for Cx43 are not remarkably altered after exposure of BREC to high glucose (data not shown), the decreased amounts of Cx43 observed under high glucose are likely to be related to an increased turnover of the protein. To determine the half-life of Cx43, cells were metabolically labeled with [^{35}S]methionine for 1 h and then chased for 1, 2, or 4 h. The data presented in Fig. 2 shows that the half-life of Cx43 in cells exposed to high glucose decreased from 2.3 h, in control cells, to 1.9 h. To further determine whether the increased turnover of Cx43 in high glucose was associated with increased degradation of the protein by a proteasome-dependent mechanism, proteins in cells were metabolically labeled with [^{35}S]methionine for 1 h and chased with unlabelled medium in the presence of the proteasome inhibitor MG132. Data shows that Cx43 degradation under high glucose is, at least in part, prevented by the proteasome inhibitor MG132 (Fig. 2) and by the more specific inhibitor lactacystin (data not shown). The half-life of Cx43 in cells grown in high glucose increased from 1.9 h to 3.4 h when the proteasome was inhibited. The fact that proteasome inhibitors extended Cx43 half-life beyond the half-life of the protein in controls indicates that proteasome has a role in constitutive degradation of Cx43 and that high glucose may create the conditions that favor increased degradation of the protein by a proteasome-dependent mechanism.

Increased Degradation of Cx43 under High Glucose Is Associated with Increased Phosphorylation of the Protein—High glucose was shown to induce PKC-mediated phosphorylation of Cx43, which in turn may stimulate protein degradation (11, 25, 26).

The effect of high glucose on phosphorylation of Cx43 induced by PKC was confirmed by incubating cells with staurosporine, an inhibitor of PKC. The decrease in the levels of Cx43 in cells exposed to high glucose was prevented, at least in part, by incubating cells with staurosporine (Fig. 3, *A* and *B*). Consistently, incubation with TPA, an activator of PKC, resulted in a decrease of about 85% in the content of Cx43 (Fig. 3*B*). These results show that high glucose may activate PKC, which, in turn, may hyperphosphorylate Cx43, leading to its increased degradation. Results obtained in our laboratory showed that the phosphorylated forms of Cx43 are preferentially degraded by a mechanism that involves the proteasome (17). To investigate whether proteasome is the mechanism leading to increased degradation of phosphorylated Cx43 under high glucose, Cx43 was immunoprecipitated from lysates of cells incubated under high glucose in the presence of proteasome inhibitors. Proteins were separated by SDS-PAGE and transferred to PVDF membranes that were probed with antibodies directed against the phosphoserine/phosphothreonine residues (Fig. 4*A*) or polyclonal antibodies directed against Cx43 (Fig. 4*B*). The ratio of phosphorylated/nonphosphorylated (P/NP) Cx43 was calculated and plotted in a graph (Fig. 4*D*). The results show that the ratio P/NP Cx43 increased 2-fold in cells incubated under high glucose and, more importantly, inhibition of the proteasome led to a 3-fold increase in the ratio of P/NP Cx43, as compared with control cells incubated under low glucose. On the other hand, the ratio of P/NP Cx43 was not significantly altered when the PKC inhibitor staurosporine was added to cells exposed to high glucose. These results suggest that phosphorylation of Cx43 in cells exposed to high glucose may act as a signal for degradation of the protein by a proteasome-dependent mechanism.

High Glucose Leads to Decreased Intercellular Communication by a Proteasome-dependent Mechanism—The functional

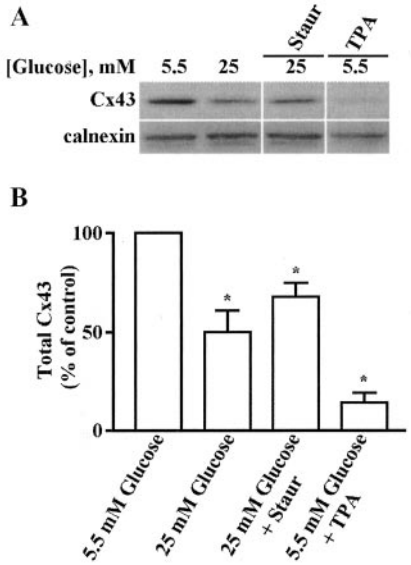

FIG. 3. **Effect of phosphorylation on the amount of Cx43.** *A*, BREC were incubated with a medium containing 5.5 or 25 mM glucose for 8 days. During the last 1 h or 40 min of incubation, cells were exposed to 100 nM staurosporine or 80 nM TPA, respectively. After the treatments, the proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with monoclonal antibodies directed against Cx43; the amount of calnexin on the same sample is included to demonstrate comparable loading of the lanes. *B*, the intensity of the bands was determined by laser scanning of the films followed by quantitative densitometric analysis; results are plotted in a graph. Data are means ± S.E. ($n = 3$ per group). *, $p < 0.05$, difference from control incubated in low glucose as determined by unpaired two-tailed Student's *t* test.

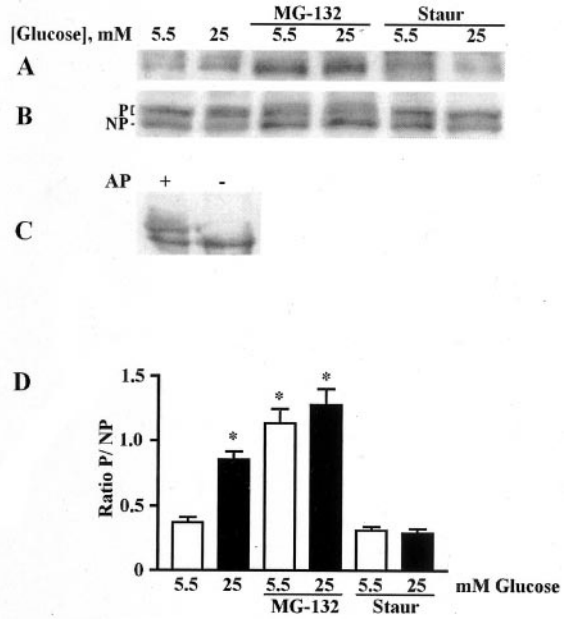

FIG. 4. **Effect of the proteasome inhibitors on phosphorylation of Cx43.** Cx43 was immunoprecipitated from cell lysates of BREC incubated with MG132 or staurosporine using polyclonal antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies directed against phosphorylated residues of serine/threonine (*A*) or polyclonal antibodies to Cx43 (*B*). *C*, enzymatic dephosphorylation of Cx43. Cell lysates were incubated in the absence or presence of alkaline phosphatase (*AP*) from *E. coli* at 37 °C overnight. The intensity of the bands was determined by laser scanning the films followed by quantitative densitometric analysis. *D*, the ratio of P/NP Cx43 was calculated and plotted in a graph. Data are means ± S.E. ($n = 3$ per group). *, $p < 0.05$, difference from control incubated in low glucose as determined by unpaired two-tailed Student's *t* test.

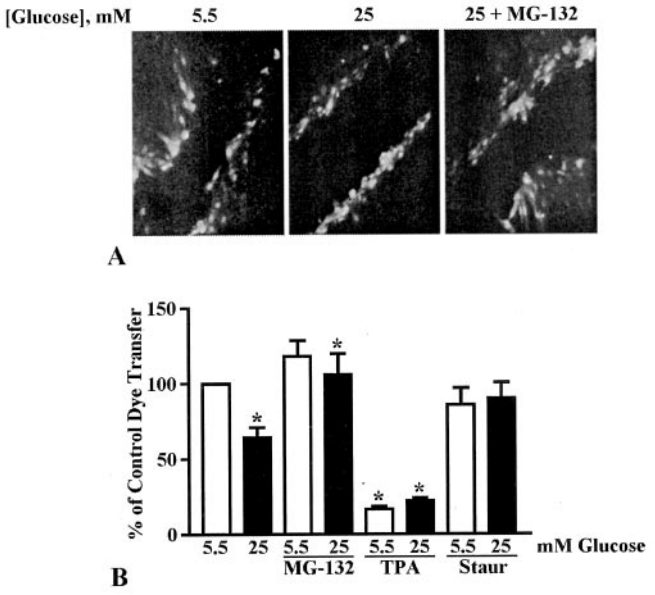

FIG. 5. **Effect of high glucose and proteasome inhibition on GJIC activity.** *A*, BREC exposed to 5.5 or 25 mM glucose for 8 days were incubated with 40 μM MG132, 100 nM staurosporine, or 80 nM TPA. The cells were then assayed for intercellular communication by Lucifer yellow dye transfer after scrape-loading. *B*, the intercellular communication was evaluated as the average distance traveled by the Lucifer yellow dye along the monolayer and is represented as a histogram. Data are means ± S.D. ($n = 3$ per group). *, $p < 0.05$, difference from control incubated in low glucose as determined by unpaired two-tailed Student's *t* test.

implications of increased Cx43 degradation in high glucose was evaluated by scrape-loading dye transfer experiments. The intercellular communication was quantified as the distance traveled by the Lucifer yellow dye after scrape-loading (Fig. 5*A*). The GJIC decreased about 40% in cells exposed to high glucose (Fig. 5*B*). Conversely, but not surprisingly, proteasome inhibitors had the opposite effect in GJIC, leading to an increase of about 20% in the distance traveled by the dye (Fig. 5*B*). However, under high glucose, proteasome inhibitors not only restored the GJIC to control levels, but also increased intercellular communication 2-fold, as compared with controls incubated under low glucose. Taken together, these results suggest that high glucose has a direct impact in GJIC by a mechanism that involves increased degradation of Cx43 in a proteasome-dependent manner.

Treatment of BREC, grown in high glucose with the PKC inhibitor staurosporine, resulted in an increase of GJIC of about 36%. On the other hand, and as predicted, treatment with TPA resulted in a dramatic inhibition (about 80%) of GJIC.

Inhibition of GJIC Is the Direct Result of Decreased Abundance of Cx43 at the Plasma Membrane—Intercellular communication is usually associated with the abundance of functional gap junction plaques, at cell-cell interfaces. Thus, an alteration of intercellular communication is either associated with changes in gap junction activity or with the levels of Cx43 at the plasma membrane available to form gap junctions. To investigate whether the decrease of GJIC in high glucose is related to alterations in the subcellular distribution of Cx43, BREC were immunostained with antibodies directed against Cx43 and imaged by confocal microscopy. In control cells, Cx43 was localized both to the plasma membrane, to cell-cell contacts, and intracellularly. In cells exposed to high glucose, there is a clear decrease in the abundance of Cx43 detected at the plasma membrane (Fig. 6*A*). The amount of Cx43 at the plasma membrane was determined as the fluorescent punctate at the site of contact between adjacent cells (Fig. 6*B*). The involve-

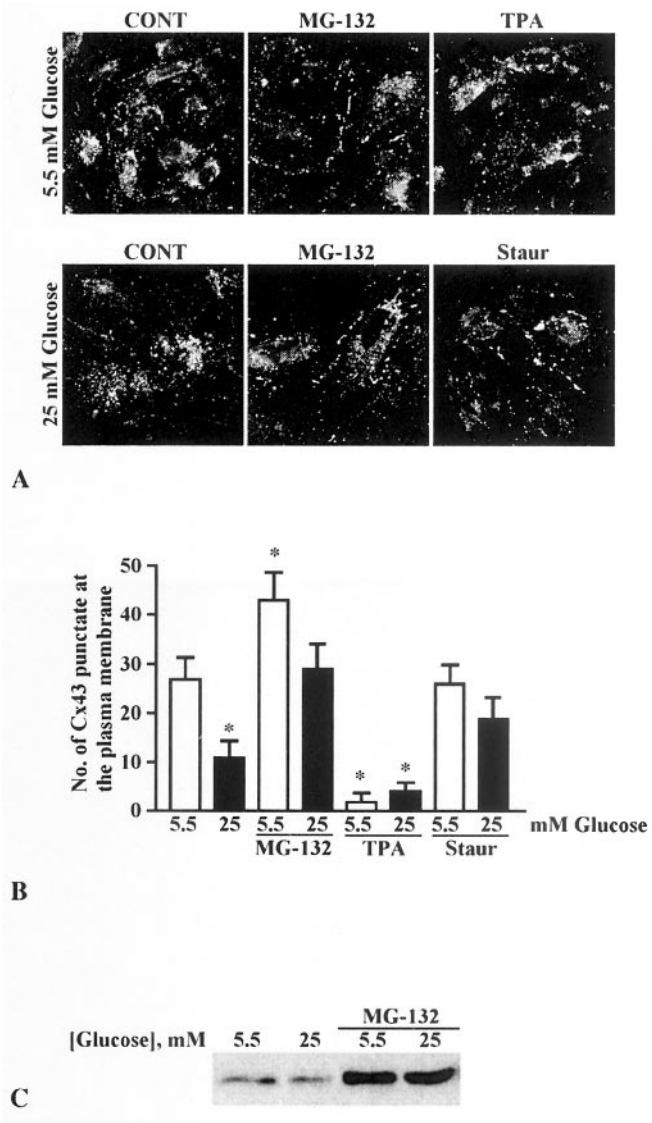

FIG. 6. **Effect of high glucose and proteasome inhibition on distribution of Cx43 at the plasma membrane.** *A*, BREC exposed to 5.5 or 25 mM glucose for 8 days were incubated with 40 μM MG132, 100 nM staurosporine, or 80 nM TPA. The cells were then fixed and stained with antibodies directed against Cx43 and imaged by confocal microscopy. *B*, the amount of Cx43 at the plasma membrane was determined as the fluorescent punctate at the site of contact between adjacent cells; results are plotted in a graph. BREC were incubated with a medium containing 5.5 or 25 mM glucose for 8 days. During the last 4 h of incubation, cells were exposed to 40 μM MG132. The cells were then surface-labeled with biotin, and the biotinylated proteins were isolated with Neutravidin beads. *C*, the isolated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with monoclonal antibodies to Cx43. Data are means ± S.D. ($n = 3$ per group). *, $p < 0.05$, difference from control incubated in low glucose as determined by unpaired two-tailed Student's t test.

ment of the proteasome on Cx43 redistribution in high glucose was determined by incubating cells with the proteasome inhibitor MG132. Results presented in Fig. 6*A* show that proteasome inhibition reversed the redistribution induced by high glucose, with the amount of Cx43, at the plasma membrane, being similar to that observed in control cells. These results indicate that inhibition of GJIC in high glucose is directly related to the amount of Cx43 available to form gap junctions at the plasma membrane. Moreover, the extraction of Cx43 from plasma membrane occurs through a proteasome-dependent mechanism and results in an increased degradation of Cx43 in retinal capillary endothelial cells.

The amount of Cx43 at the plasma membrane was further assessed by biotinylation of cell-surface proteins. The amount of biotinylated Cx43 decreased in cells exposed to high glucose, as compared with control cells (Fig. 6*C*). However, treatment with MG132 resulted in a significant increase in the amount of biotinylated Cx43 recovered in lysates of BREC exposed to high glucose (Fig. 6*C*).

Taken together, these results support a model in which hyperglycemia creates the conditions that favor targeting of membrane Cx43 to degradation by a proteasome-dependent mechanism. The results presented in this study, together with previous reports, suggest that phosphorylation of Cx43 is the stimulus targeting Cx43 for degradation by a proteasome-dependent mechanism. Moreover, increased degradation of Cx43 results in a decrease in the amount of Cx43 available to form gap junctions at the plasma membrane, leading to a reduction in intercellular communication in endothelial cells.

DISCUSSION

Previous studies demonstrated that high glucose induces a reduction of GJIC (25–28). More recently, Sato *et al.* (11) showed that the decreased levels of Cx43 were partially associated with a down-regulation of Cx43 in response to hyperglycemia. Endothelial cells exposed to high glucose showed a decrease of about 30% in Cx43 mRNA, whereas the total amount of the protein decreased 45%, what suggests that Cx43 degradation might also be enhanced in hyperglycemia (11). Data reported in this study show that the amount of Cx43 in BREC decreased about 50% in high glucose, whereas the levels of Cx43 mRNA do not vary significantly. We have shown previously (17) in other cell types that in addition to the lysosome, Cx43 abundance can be regulated by a ubiquitin-proteasome-dependent mechanism. Thus, we hypothesized that the reduction of Cx43 induced by high glucose might be associated to an increase in the turnover of the protein. In support of this hypothesis is the observation that the half-life of Cx43 in BREC exposed to high glucose decreased about 20% (this study). Most likely, the lysosome is the major site for constitutive degradation of Cx43. However, it has been well established that proteasome is also involved in degradation of Cx43 in a number of cell types (13–17). Data obtained in this study show, for the first time, that the proteasome is involved in degradation of Cx43 in blood retinal endothelial cells in high glucose. Significantly, we have further shown that degradation of Cx43 by a proteasome-dependent mechanism can be enhanced by protein phosphorylation and results in decreased GJIC (17). The evidence for proteasomal degradation of Cx43 is such that it has been considered a novel mechanism for regulating intercellular communication (14, 17). It has been shown that phosphorylation of Cx43 by various kinases, including PKC (23, 34), mitogen-activated protein kinase (24, 29), and the v-Src tyrosine protein kinase (29, 30), all result in decreased intercellular communication. We have suggested before (17) that phosphorylation of Cx43 is the signal that targets Cx43 for degradation by a proteasome-dependent mechanism.

The pathways that transduce hyperglycemia into increased degradation of Cx43 are largely unknown, as are the triggering signals and the mechanism for extraction of Cx43 from plasma membrane. However, by analogy to what has been observed in other cell systems and based upon accepted models for regulation of GJIC, it is reasonable to suggest that phosphorylation of Cx43 is the upstream signaling event that triggers the degradation of the protein by a proteasome-dependent mechanism. Indeed, we show in the present report that activation of PKC is required to phosphorylate Cx43 in cells exposed to high glucose, as revealed by the inhibitory effect of staurosporine. There is ample evidence in the literature to suggest that hy-

perglycemia and diabetes are associated with increased activity of PKC (25, 26, 31–33). Moreover, hyperphosphorylation of Cx43 in conditions of hyperglycemia has been widely demonstrated (25, 26, 32). Multiple isoforms of PKC, including PKC α, β1, β2, and δ, were shown to be activated in response to high glucose (34–36). More importantly, activation of various isoforms of PKC is implicated in vascular dysfunction associated with diabetes (35–37). At least part of these isoforms were shown to phosphorylate Cx43 (38–40), presumably targeting the protein for degradation (17). At present, the specific isoforms that lead to increased phosphorylation of Cx43 in high glucose, and presumably in diabetes, remain to be elucidated. However, data presented in this report clearly show an association between exposure of retinal endothelial cells to high glucose, hyperphosphorylation of Cx43, and increased degradation of the protein by a proteasome-dependent mechanism.

Consistent with the above model, we also demonstrate that inhibition of the proteasome leads to an accumulation of phosphorylated Cx43. These data, together with the observation that staurosporine (an inhibitor of PKC) and TPA (an activator of PKC) have opposite effects upon the half-life of the protein, strongly suggest that phosphorylation of Cx43 leads to its extraction from the plasma membrane and increased degradation.

The physiological implications of hyperglycemia in GJIC have been extensively demonstrated in various cell types and systems. For example, in retinal pigment epithelium (41), epididymal endothelial cells (11, 27), and smooth muscle cells (25), it has been shown that high glucose reduced the intercellular communication. Significantly, the decrease in intercellular communication induced by high glucose, in most studies, can be reversed by PKC inhibitors (25, 28, 32), thus indicating that phosphorylation is the mechanism whereby hyperglycemia reduces intercellular communication.

Data presented in this study consistently show that high glucose reduces GJIC and also that such reduction involves the activation of PKC and, most likely, other kinases (because the intercellular communication is not completely reversed by PKC inhibitor). More importantly, we propose an integrated model in which increased phosphorylation of Cx43 (mostly by PKC) under high glucose leads to extraction of Cx43 from the plasma membrane and increased degradation of Cx43, with the consequent reduction of GJIC. To demonstrate that reduction of GJIC is the direct result of the decreased abundance of Cx43 available to form gap junction plaques and not the result of alterations on the activity of gap junctions, we performed biotinylation of membrane proteins and imaged cell cultures by confocal microscopy. Data obtained confirm that high glucose leads to a reduction on the abundance of Cx43 forming gap junctions at the plasma membrane and that such reduction can be prevented by inhibitors of the proteasome.

Taken together, the data presented in this report support a model in which hyperphosphorylation of Cx43 in hyperglycemia constitutes the triggering signal for Cx43 degradation by a proteasome-dependent mechanism. We suggest that impairment of gap junction intercellular communication activity between vascular endothelial cells is involved in the breakdown of the blood-retinal barrier in chronic hyperglycemia (11). Increased degradation of Cx43 and reduction of GJIC may thus contribute to the endothelial cell dysfunction associated with breakdown of the blood-retinal barrier associated with diabetic retinopathy.

REFERENCES

1. Winegrad, A. I. (1987) *Diabetes* **36,** 396–406
2. King, G. L., Kunisaki, M., Nishio, Y., Inoguchi, T., Shiba, T., and Xia, P. (1996) *Diabetes* **45,** S105–S108
3. Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I., and Brownlee, M. (2000) *Nature* **404,** 787–790
4. Obrosova, I. G., Minchenko, A. G., Vasupuram, R., White, L., Abatan, O. I., Kumagai, A. K., Frank, R. N., and Stevens, M. J. (2003) *Diabetes* **52,** 864–871
5. Koya, D., and King, G. L. (1998) *Diabetes* **47,** 859–866
6. Kumar, N. M., and Gilula, N. B. (1996) *Cell* **84,** 381–388
7. Simon, A. M., and Goodenough, D. A. (1998) *Trends Cell* **8,** 477–483
8. Yeh, H. I., Rothery, S., Dupont, E., Coppen, S. R., and Severs, N. J. (1998) *Circ. Res.* **83,** 1248–1263
9. Little, T. L., Beyer, E. C., and Duling, B. R. (1995) *Am. J. Physiol.* **268,** H729–H739
10. Larson, D. M., Wrobleski, M. J., Sagar, G. D., Westphale, E. M., and Beyer, E. C. (1997) *Am. J. Physiol.* **272,** C405–C415
11. Sato, T., Haimovici, R., Kao, R., Li, A. F., and Roy, S. (2002) *Diabetes* **51,** 1565–1571
12. Fallon, R. F., and Goodenough, D. A. (1981) *J. Cell Biol.* **90,** 521–526
13. Beardslee, M., Lerner, D., Tadros, P., Laing, J., Beyer, E., Yamada, K., Kleber, A., Schuessler, R., and Saffitz, J. (2000) *Circ. Res.* **87,** 656–662
14. Musil, L. S., Le, A. C., Van Slyke, J. K., and Roberts, L. M. (2000) *J. Biol. Chem.* **275,** 25207–25215
15. Laing, J. G., Tadros, P. N., Westphale, E. M., and Beyer, E. C. (1997) *Exp. Cell Res.* **236,** 482–492
16. Laing, J. G., and Beyer, E. C. (1995) *J. Biol. Chem.* **270,** 26399–26403
17. Girao, H., and Pereira, P. (2003) *Mol. Vis.* **9,** 24–30
18. Fuertes, G., Martin De Llano, J. J., Villarroya, A., Rivett, A. J., and Knecht, E. (2003) *Biochem. J.* **375,** 75–86
19. Qin, H., Shao, Q., Igdoura, S. A., Alaoui-Jamali, M. A., and Laird, D. W. (2003) *J. Biol. Chem.* **278,** 30005–30014
20. Ward, W. F. (2002) *Prog. Mol. Subcell. Biol.* **29,** 35–42
21. Matesic, D., Rupp, H., Bonney, W., Ruch, R., and Trosko, J. (1994) *Mol. Carcinog.* **10,** 226–236
22. TenBroek, E., Louis, C., and Jonhson, R. (1997) *Dev. Biol.* **191,** 88–102
23. Lampe, P. D., TenBroek, E. M., Burt, J. M., Kurata, W. E., Johnson, R. G., and Lau, A. F. (2000) *J. Cell Biol.* **149,** 1503–1512
24. Rivedal, E., and Opshal, H. (2001) *Carcinogenesis* **22,** 1543–1550
25. Kuroki, T., Inoguchi, T., Umeda, F., Ueda, F., and Nawata, H. (1998) *Diabetes* **47,** 931–936
26. Malfait, M., Gomez, P., van Veen, T. A., Parys, J. B., De Smedt, H., Vereecke, J., and Himpens, B. (2001) *J. Membr. Biol.* **181,** 31–40
27. Inoguchi, T., Ueda, F., Umeda, F., Yamashita, T., and Nawata, H. (1995) *Biochem. Biophys. Res. Commun.* **208,** 492–497
28. Stalmans, P., and Himpens, B. (1997) *Invest. Ophthalmol. Vis. Sci.* **38,** 1598–1609
29. Cottrell, G. T., Lin, R., Warn-Cramer, B. J., Lau, A. F., and Burt, J. M. (2003) *Am. J. Physiol.* **284,** C511–C520
30. Lin, R., Warn-Cramer, B. J., Kurata, W. E., and Lau, A. F. (2001) *J. Cell Biol.* **154,** 815–827
31. Inoguchi, T., Battan, R., Handler, E., Sportsman, J. R., Heath, W., and King, G. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11059–11063
32. Inoguchi, T., Yu, H. Y., Imamura, M., Kakimoto, M., Kuroki, T., Maruyama, T., and Nawata, H. (2001) *Med. Electron Microsc.* **34,** 86–91
33. Shiba, T., Inoguchi, T., Sportsman, J. R., Heath, W. F., Bursell, S., and King, G. L. (1993) *Am. J. Physiol.* **265,** E783–E793
34. Park, J. Y., Takahara, N., Gabriele, A., Chou, E., Naruse, K., Suzuma, K., Yamauchi, T., Ha, S. W., Meier, M., Rhodes, C. J., and King, G. L. (2000) *Diabetes* **49,** 1239–1248
35. Babazono, T., Kapor-Drezgic, J., Dlugosz, J. A., and Whiteside, C. (1998) *Diabetes* **47,** 668–676
36. Koya, D., Jirousek, M. R., Lin, Y. W., Ishii, H., Kuboki, K., and King, G. L. (1997) *J. Clin. Invest.* **100,** 115–126
37. Ishii, H., Jirousek, M. R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S. E., Kern, T. S., Ballas, L. M., Heath, W. F., Stramm, L. E., Feener, E. P., and King, G. L. (1996) *Science* **272,** 728–731
38. Schulz, R., Gres, P., Skyschally, A., Duschin, A., Belosjorow, S., Konietzka, I., and Heusch, G. (2003) *FASEB J.* **17,** 1355–1357
39. Bowling, N., Huang, X., Sandusky, G. E., Fouts, R. L., Mintze, K., Esterman, M., Allen, P. D., Maddi, R., McCall, E., and Vlahos, C. J. (2001) *J. Mol. Cell Cardiol.* **33,** 789–798
40. Husoy, T., Cruciani, V., Sanner, T., and Mikalsen, S. O. (2001) *Carcinogenesis* **22,** 221–231
41. Himpens, B., Stalmans, P., Gomez, P., Malfait, M., and Vereecke, J. (1999) *FASEB J.* **13,** S63–S68